Inhibition of insulin release by synthetic peptides shows that the H3 region at the C-terminal domain of syntaxin-1 is crucial for Ca^{2+}- but not for guanosine 5′-[γ-thio]triphosphate-induced secretion

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Recently, we have described the presence and possible role of syntaxin in pancreatic β-cells by using monoclonal antibodies [F. Martin, F. Moya, L. M. Gutierrez, J. A. Reig, B. Soria (1995) Diabetologia **38**, 860–863]. In order to characterize further the importance of specific domains of this protein, the functional role of a particular region of the syntaxin-1 molecule has now been investigated by using two synthetic peptides, SynA and SynB, corresponding to two portions of the H3 region at the C-terminal domain of the protein, residues 229–251 and 197–219 respectively. Functional experiments carried out in permeabilized pancreatic β-cells demonstrate that these peptides inhibit Ca^{2+}- dependent insulin release in a dose-dependent manner. This effect is specific because peptides of the same composition but random sequence do not show the same effect. In contrast with this inhibitory effect on Ca^{2+}-induced secretion, both peptides increase basal release. However, under the same conditions, SynA and SynB do not affect guanosine 5′-[γ-thio]triphosphate-induced insulin release. These results demonstrate that specific portions of the H3 region of syntaxin-1 are involved in critical protein–protein interactions specifically during Ca^{2+}-induced insulin secretion.

INTRODUCTION

The release of insulin from pancreatic β-cells is induced by glucose via an increase in intracellular Ca^{2+} concentration. This specific effect of glucose and other metabolic nutrients is caused by the closure of ATP-dependent K^+ channels [1,2]. Recent results indicate that the molecular events and intracellular proteins that underlay this process are shared by other secretory cells, including the synaptic terminal, in which the current hypothesis concerning the process of regulated exocytosis has been established [3,4]. Briefly, this model proposes that the exocytotic process is a generalized mechanism which involves the interaction of specific proteins, including synaptobrevin, also termed vesicle-associated membrane protein (VAMP), present in the secretory vesicle, and other proteins in the plasma membrane such as 25 kDa synaptosomal-associated protein (SNAP25) and syntaxin [5]. The sequential binding of soluble proteins, identified as *N*-ethylmaleimide-sensitive fusion protein (NSF) and α-soluble NSF-attachment protein (α-SNAP) [6,7], to the initial protein complex finally induces membrane fusion and secretion. Another protein present in the vesicle, synaptotagmin, may be the negative regulatory component of this process allowing vesicle docking in the absence of Ca^{2+}. Although specific intracellular target(s) for Ca^{2+} and consequently the molecular switch for secretion has not yet been established, the presence in the structure of synaptotagmin of two copies of the C2 regulatory region of protein kinase C suggests that this protein may have an important role in the Ca^{2+}-induced secretory pathway acting as a Ca^{2+} sensor [8].

Syntaxin is currently considered to be a pivotal protein in the sequential mechanism of exocytosis [9]. It apparently interacts with at least five proteins during the secretory process: VAMP, SNAP25, n-sec, α-SNAP and N-type Ca^{2+} channels [10,11]. Experiments using deletions and point mutations have recently established the relative importance of three regions of the syntaxin molecule, defined as H1, H2 and H3 [12], in the interaction of this protein with presynaptic elements involved in the secretory process. The putative helical domain, H3, was shown to be critical for syntaxin to bind to α-SNAP, SNAP25 and VAMP. However, a functional correlation of the importance of H3 as well as other regions of the syntaxin molecule with secretion has not yet been addressed.

The presence in endocrine systems of different proteins involved in the formation of the fusion complex, initially reported at the presynaptic terminal, has been demonstrated [13,14], supporting the idea of a general mechanism for regulated exocytosis. Syntaxin-1 has been characterized in nervous tissue [15] as well as in pancreatic β-cells [16], where specific anti-syntaxin antibodies have been shown to partially inhibit insulin release.

The possibility of uncoupling the secretory process by using peptides that resemble specific regions of proteins involved in exocytosis, such as VAMP [17] and SNAP25 [18] which are specific substrates of proteolytic neurotoxins, has initially permitted a partial explanation of the mode of action of tetanus and botulinum toxins. These toxins induce the release of soluble fragments of these proteins into the cytoplasm and these peptides compete with endogenous substrates leading to an alteration of the secretory process. In addition, the use of synthetic peptides has provided an interesting tool for the study of the functional role of specific domains of proteins supposedly implicated in insulin release [19].

Exocytosis is not only activated by an increase in intracellular Ca^{2+} concentration, but also by the action of GTP-binding proteins, which have also been implicated in secretion induced in the absence of Ca^{2+}, by using hydrolysis-resistant analogues of GTP in different systems [20], including the insulin-secreting cells HIT-T15 [21,22]. However, this result has not yet been confirmed

Abbreviations used: VAMP, vesicle-associated membrane protein; SNAP25, 25 kDa synaptosomal-associated protein; NSF, *N*-ethylmaleimide-sensitive fusion protein; α-SNAP, α-soluble NSF-attachment protein.

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in normal pancreatic β-cells. The effect of GTP analogues may be related to the activation of both low-molecular-mass GTP-binding proteins [19] and heterotrimeric GTP-binding proteins, which have been reported in both plasma and granule membranes [23].

Our aim in the present work was to investigate whether synthetic peptides, corresponding to two non-overlapping zones of the defined H3 region of syntaxin-1, modify insulin secretion stimulated by either Ca^{2+} or guanosine 5′-[γ-thio]triphosphate (GTP[S]) in permeabilized mouse pancreatic β-cells.

MATERIALS AND METHODS

Materials

Collagenase was from Boehringer-Mannheim (Mannheim, Germany). Tissue culture reagents were from Cultek (Madrid, Spain). GTP[S] and the monoclonal anti-syntaxin antibody (clon HPC-1) were from Sigma (Madrid, Spain).

Cell isolation, permeabilization and insulin secretion

Adult (8–10-week-old) male Swiss mice (OF1) (CRIFFA, Barcelona, Spain) were used throughout this study. Pancreatic islet cells (4.5×10^5) were dispersed from isolated islets and then permeabilized by using 10 μM digitonin at 37 °C for 5 min in a Hepes medium (25 mM Hepes, 110 mM KCl, 10 mM NaCl, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 5 mM succinate and 1 mg/ml BSA) as previously described [16]. During the permeabilization period (5 min) islet cells were incubated in the absence or presence of different peptides at the indicated concentrations. After permeabilization, insulin release was studied by incubating cells for 10 min in the absence or presence of the different peptides in the same Hepes buffer but free of digitonin and supplemented with 2 mM ATP and an ATP-regenerating system consisting of 15 mM phosphocreatine and 20 units/ml creatine kinase. Two different buffered free Ca^{2+} concentrations [50 nM (basal secretion) and 10 μM (Ca^{2+}-stimulated secretion)] were used in either the absence or presence of the different peptides. Secretion was also induced with 50 nM free Ca^{2+} in the presence of different concentrations of GTP[S]. Finally, the effect of the syntaxin-related peptides was studied in permeabilized β-cells stimulated with 100 μM GTP[S]. Insulin released was determined by RIA using the kit provided by DPC (Los Angeles, CA, U.S.A.). None of the peptides studied at concentrations higher than those utilized in the experiments modified the standard insulin calibration curve, thus the different peptides used in the present study did not interfere with the IRA determinations.

Peptide synthesis and purification

Peptides synthesized corresponding to the H3 syntaxin fragment and spanning the specified residues were (Figure 1): Syn229–251 (SynA) with the sequence MIDRIEYNVEHSVDYVERAVSDT and Syn197–219 (SynB) with the sequence TRHSEIIKLENSIRELHDMFMDM. Two control peptides, ConA and ConB, contained the same amino acids but in random order, DYRTRVEMDNDVAIVHISYEVSE and EILLKHSMRIEFDNSTMRHEIMD respectively. Peptides were synthesized in the Protein Chemistry Facility of the Centro Biologia Molecular 'Severo Ochoa' (Madrid, Spain) using Fmoc chemistry in an ABI-431A peptide synthesizer (Applied Biosystems). The purity of the peptides (higher than 95 %) was checked by HPLC analysis and exact peptide identity was confirmed by MS. The molecular masses of SynA and SynB were 2742.2 and 2847.5 respectively.

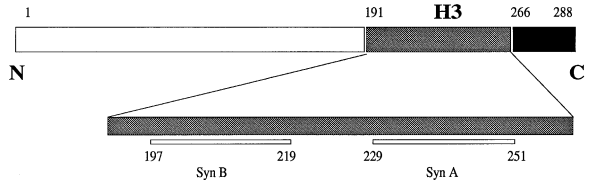

Figure 1 Schematic diagram of syntaxin protein

H3 region and the relative position of the sequences of SynA and SynB are illustrated. The transmembrane domain at the C-terminus is also indicated by a filled segment.

Statistics

Results are presented as means ± S.E.M. Statistical analysis was performed by Student's two tailed t test for unpaired data, and $P < 0.05$ was considered to be significant.

RESULTS

Effect of SynA and SynB on basal and Ca^{2+}-induced insulin release

The H3 region of the syntaxin-1 molecule corresponding to amino acids 191–265 has been shown to be important in the interactions of syntaxin with other proteins that participate in the secretory process [12]. In order to investigate the functional relevance of this apparently unique syntaxin domain, and to identify which portions of H3 constitute the specific protein recognition domains during secretion, we synthesized two 23-mer peptides, SynA (amino acids 229–251) and SynB (amino acids 197–219), which correspond to non-overlapping sequences spanning more than 60 % of the H3 region (Figure 1). Experiments were carried out with digitonin-permeabilized β-cells which were incubated with the peptides for 5 min before the secretory stimulus, which was applied for an additional 10 min in supplemented Hepes buffer in the presence or absence of the peptides, as indicated in the Materials and methods section. Owing to the

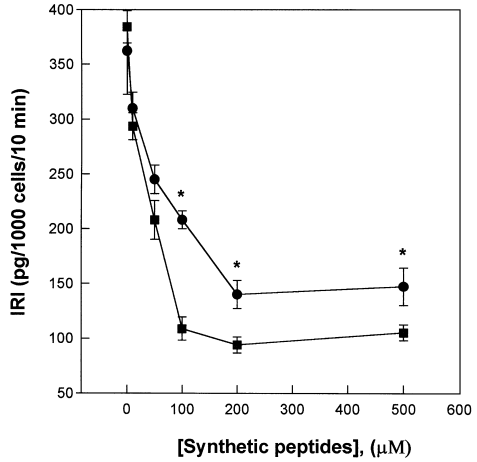

Figure 2 Dose-dependent effect of SynA and SynB on Ca^{2+}-induced insulin release

Pancreatic β-cells were permeabilized for 5 min with digitonin and incubated in either the presence or absence of different concentrations of SynA (●) or SynB (■). Stimulation with 10 μM Ca^{2+} was further performed in a digitonin-free medium for 10 more minutes in the presence of the different concentrations of the peptides assayed. IRI, immunoreactive insulin. $^*P < 0.05$ when compared with SynB ($n = 4$).

Table 1 Specificity of the effect of SynA and SynB on Ca^{2+}-induced insulin release

Pancreatic β-cells were permeabilized for 5 min with digitonin and incubated in the absence or presence of the syntaxin-related peptides (SynA and SynB) or the control peptides with random sequences (ConA and ConB respectively) (all at 200 μM). When two peptides were assayed together (SynA + SynB or ConA + ConB) a 100 μM concentration of each was used. The medium was then changed and insulin secretion was triggered by incubation with a 10 μM Ca^{2+} solution for 10 more minutes, in both the absence (control) and presence of the different peptides at the same concentrations and in a digitonin-free medium. The 2*P* values are compared with controls; NS, not significant.

Agents added during incubation	Immunoreactive insulin (pg/1000 cells per 10 min)		
	Mean ± S.E.M.	*n*	2*P*
Control	387.16 ± 8.42	4	
SynA (200 μM)	140.17 ± 5.83	6	< 0.0001
ConA (200 μM)	360.39 ± 25.90	5	NS
SynB (200 μM)	111.94 ± 7.39	3	< 0.0001
ConB (200 μM)	315.63 ± 10.64	4	NS
SynA (100 μM) + SynB (100 μM)	84.01 ± 5.66	5	< 0.0001
ConA (100 μM) + ConB (100 μM)	311.43 ± 31.80	5	NS

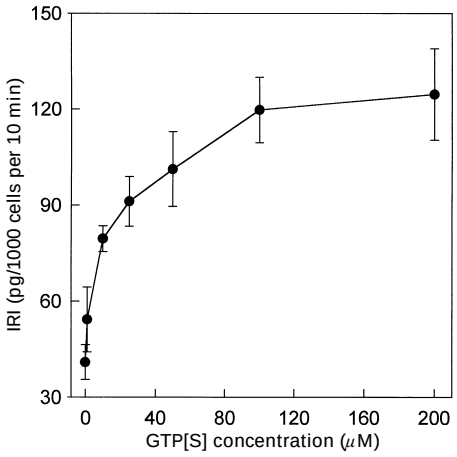

Figure 3 Insulin secretion induced by GTP[S]

Pancreatic β-cells were permeabilized for 5 min with digitonin and incubated for a further 10 min in the presence of the indicated concentrations of GTP[S] in a digitonin-free solution containing 50 nM buffered free Ca^{2+}. $n = 5$. IRI, immunoreactive insulin.

previously reported [19] 'run-down' effect in digitonin-permeabilized cells, the induced secretory response decreases with incubation time after permeabilization. A 10 min incubation period was shown to be optimal [16], considering the ratio between basal and stimulated secretion, and therefore this was used in the present study. Figure 2 shows the dose-dependent inhibitory effects of SynA and SynB on Ca^{2+}-induced release. Both peptides decreased the Ca^{2+}-induced insulin release, the effect of SynB being significantly higher ($P < 0.05$ when compared with SynA at 100, 200 and 500 μM). The IC_{50} values were 46 and 32 μM for SynA and SynB respectively; in both cases maximal effects were reached at 200 μM and represented 63 ± 6 % and 73 ± 8 % of inhibition respectively. This effect was caused by an intracellular action of these peptides, because they did not modify glucose-induced insulin release in non-permeabilized cells (results not shown).

Table 2 Specificity of the effect of SynA and SynB on basal insulin release

Pancreatic β-cells were permeabilized for 5 min with digitonin and incubated in the absence or presence of the syntaxin-related peptides (SynA and SynB) or the control peptides with random sequences (ConA and ConB respectively) (all at 200 μM). When two peptides were assayed together (SynA + SynB or ConA + ConB) a 100 μM concentration of each was used. The medium was then changed and cells were incubated in 50 nM Ca^{2+} solution for 10 more min, in both the absence (control) and presence of the different peptides at the same concentrations and in a digitonin-free medium. The 2*P* values are compared with controls; NS, not significant.

Agents added during incubation	Immunoreactive insulin (pg/1000 cells per 10 min)		
	Mean ± S.E.M.	*n*	2*P*
Control	59.06 ± 1.88	4	
SynA (200 μM)	68.13 ± 1.71	5	< 0.01
ConA (200 μM)	45.58 ± 3.83	6	NS
SynB (200 μM)	84.12 ± 3.61	4	< 0.002
ConB (200 μM)	51.90 ± 3.44	NS	
SynA (100 μM) + SynB (100 μM)	81.09 ± 4.56	5	< 0.003
ConA (100 μM) + ConB (100 μM)	47.25 ± 7.22	4	NS

In order to investigate the specificity of this inhibition, we used control synthetic peptides, with the same composition as SynA and SynB but with a random sequence, termed ConA and ConB respectively. The sequences of these control peptides are specified in the Materials and methods section. Table 1 shows an experiment in which the same concentration (200 μM) was used for the four peptides. Control peptides had no significative effect on Ca^{2+}-induced secretion in contrast with the marked inhibition caused by SynA (64 ± 7 %) and SynB (70 ± 6 %) ($P < 0.0001$ when compared with control). When SynA and SynB were incubated together at a concentration of 100 μM each, we observed a higher level of inhibition (79 ± 8 %) ($P < 0.0001$ when compared with control), but this value was not significantly different from the results obtained separately with each peptide.

Interestingly, under the assay conditions, basal secretion in the presence of 50 nM free Ca^{2+} was also slightly, but significantly, affected by SynA and SynB (Table 2). The increase amounted to 33 ± 3 % and to 64 ± 8 % with SynA and SynB respectively ($P < 0.01$ and $P < 0.002$ respectively when compared with control) whereas control peptides did not affect basal release, indicating that this effect was also specific. When SynA and SynB were incubated together, no additive effect was observed.

GTP[S]-induced insulin release

The reported effect of non-hydrolysable GTP analogues on insulin secretion in insulin-secreting HIT-T15 cells [21,22] prompted us to investigate the effect of GTP[S] on insulin release in normal mouse pancreatic β-cells. Figure 3 shows the dose–response stimulatory effect of this non-hydrolysable analogue on insulin secretion at 50 nM free buffered Ca^{2+}. GTP[S] induced the release of insulin with an EC_{50} of 50 μM. The maximal secretion induced at 100 μM amounted to approx. 29 % compared with the secretion induced by 10 μM free Ca^{2+}. These values are very close to those previously reported for insulin-secreting HIT-T15 cells [19,21]. Experiments were then performed to investigate whether syntaxin was involved in this secretory mechanism. In the first place, we tested the effects of SynA and SynB and observed that neither modified the secretory response (Table 3), demonstrating that the H3 region of syntaxin is involved in Ca^{2+}-induced, but apparently not with GTP[S]-

Table 3 Absence of the effect of SynA and SynB on GTP[S]-induced insulin release

Pancreatic β-cells were permeabilized for 5 min with digitonin and incubated in the absence or presence of the syntaxin-related peptides (SynA and SynB) or the control peptides with random sequences (ConA and ConB respectively) (all at 200 μM). When two peptides were assayed together (SynA + SynB or ConA + ConB) a 100 μM concentration of each was used. The anti-syntaxin antibody clon HPC-1 was used at a dilution of 1:250. The medium was then changed and insulin release was induced for a further 10 min in a 50 nM Ca^{2+} solution in the presence of 100 μM GTP[S] (except in the control), in a digitonin-free medium. Both the peptides and antibody were also present during the stimulation at the same concentrations. The 2*P* values are compared with results obtained with 100 μM GTP[S]; NS, not significant.

Agents added during incubation	Immunoreactive insulin (pg/100 cells per 10 min)		
	Mean ± S.E.M.	*n*	2*P*
Control	51.06 ± 5.37	4	
GTP[S] (100 μM)	112.60 ± 9.51	4	
HPC-1	126.83 ± 8.23	4	
SynA (200 μM)	112.24 ± 7.73	5	NS
ConA (200 μM)	114.61 ± 4.95	4	NS
SynB (200 μM)	118.70 ± 11.46	4	NS
ConB (200 μM)	106.29 ± 7.59	5	NS
SynA (100 μM) + SynB (100 μM)	114.26 ± 6.72	4	NS
ConA (100 μM) + ConB (100 μM)	98.01 ± 10.48	4	NS

induced, secretion. In a previous report we have shown that anti-syntaxin monoclonal antibodies partially inhibit Ca^{2+}-induced insulin release. Therefore it was also of interest to investigate the possible effect of this antibody on the secretion induced by non-hydrolysable analogues of GTP. As shown in Table 3, secretion induced by 100 μM GTP[S] was not significantly affected by this antibody (clon HPC-1), supporting the idea that syntaxin is not involved in this secretory mechanism.

DISCUSSION

The results of the present study demonstrate that two specific peptides, SynA and SynB, synthesized according to the sequence of two segments of the H3 region (residues 191–266) of syntaxin [12], specifically inhibit Ca^{2+}-induced insulin release, thereby establishing for the first time a functional correlation between the H3 region of syntaxin and the secretory process. Although we have no direct evidence, the most probable cause of the inhibitory effect of SynA and SynB is competition of these peptides with endogenous syntaxin targets. Thus SynA and SynB may inhibit Ca^{2+}-induced secretion by uncoupling the binding of syntaxin to other proteins involved in the subsequent exocytotic mechanism. We can rule out non-specific effects, since control peptides had no effect on secretion and also because of the absence of any effect of SynA and SynB in intact cells.

Our data corroborate previous results showing that monoclonal anti-syntaxin antibodies inhibit Ca^{2+}-induced insulin release [16], and therefore suggest a crucial role for this protein in the secretory pathway in pancreatic β-cells. Besides the role of syntaxin in the docking and fusion of secretory granules, which is generally assumed in the current hypothesis on regulated exocytosis [3,4], an additional negative role of syntaxin has also been recently proposed from transfection experiments with mouse BTC3 cells. In that study a reduction in glucose-induced insulin secretion was reported after syntaxin-1A overexpression [24]; however, in this case insulin secretion was measured after 2 days of continuous glucose treatment and therefore other parallel effects induced by glucose cannot be ruled out. In any case, it is evident that the role of a syntaxin in secretion should be carefully considered because of its multiple sequential protein–protein interactions.

In vitro studies have implicated the H3 region of syntaxin in the binding of SNAP25, α-SNAP and VAMP, probably through the interaction of coiled-coil motifs of the different proteins. The binding of SNAP25 to syntaxin was demonstrated to be the less restrictive and was shown to require only the presence of amino acids 191–221, which correspond to the end segment of H3 closest to the N-terminus of syntaxin [12]. In fact, previous results had already demonstrated the involvement of a longer sequence corresponding to residues 199–243 in the interaction of syntaxin with SNAP25 [25]. SynB (amino acids 197–219) comprises more than 75% of this portion of H3 (Figure 1) and therefore it may be interacting with the specific domain(s) of SNAP25 that binds to syntaxin. This interaction would probably diminish the faculty of SNAP25 to bind to this protein. On the other hand, the interaction of syntaxin with α-SNAP and VAMP requires additional amino acids located at the C-terminal extreme of H3 [12]. Since SynA (amino acids 229–251) spans the C-terminal segment of H3 (Figure 1), it may be interacting with either of these two proteins preventing the formation of an operative protein complex. We cannot rule out other intracellular targets for these two peptides.

The absence of any effect of SynA and SynB on GTP[S]-induced secretion supports the evidence that syntaxin is not essential for this mechanism. Recently, it has been reported that VAMPs are not involved in GTP[S]-induced secretion in HIT-T15 cells [26] and that, in contrast with the Ca^{2+}-induced release which displayed run-down in digitonin-permeabilized cells, the release of insulin induced by the non-hydrolysable analogue of GTP was kept constant over the entire range studied [27]. Our results reinforce the idea that the mechanisms of Ca^{2+}- and GTP[S]-induced insulin release in pancreatic β-cells involve different proteins.

In contrast with the stimulated secretion, basal release of insulin was increased by SynA and SynB. It is probable that these peptides initially uncouple the postulated negative regulatory role of syntaxin that prevents secretion under basal conditions, thereby allowing an increase in the basal release. Our results support the idea that the docking of granules is initially established by syntaxin in a heterotrimeric complex in which the role of syntaxin can be defined at two different stages: during the docking step and in the Ca^{2+}-induced fusion mechanism. Apparently, the synthetic peptides studied interfere with both events in the absence and presence of Ca^{2+}. In the docking complex, a possible negative action of syntaxin can be overcome by the synthetic peptides through interaction with SNAP25 and VAMP which consequently leads to an increase in basal release. On the other hand, Ca^{2+}-induced secretion would be inhibited by the peptides through competition with additional endogenous targets such as α-SNAP.

In conclusion, these results support the hypothesis that the H3 region of the syntaxin molecule is critical for Ca^{2+}-induced insulin release but not for GTP[S]-triggered secretion. In addition, these results demonstrate that specific peptides as short as 23 residues themselves possess possible binding properties inherent to the H3 region that allow uncoupling of the secretory process. Further systematic studies are required to determine the possible intracellular targets of these peptides as well as the minimum active domains of syntaxin directly involved in its function in pancreatic β-cells.

We thank Nuria Illera and Rosa Garcia for their help in the preparation of mouse pancreatic β-cells. These studies were supported by grants GV-2518/94 from

Generalidad Valenciana to J.A.R., grant FIS 94-0014-01 from Fondo de Investigaciones Sanitarias to B.S., grant ERBSC1-CT 920833 from the European Union to B.S. and grant GV-3117/95 from Generalidat Valenciana to B.S. and J.A.R.

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Received 30 May 1996/22 July 1996; accepted 24 July 1996